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# Excitotoxic stimulation downregulates the ubiquitin–proteasome system through activation of NMDA receptors in cultured hippocampal neurons

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ABSTRACT

Overactivation of glutamate receptors contributes to neuronal damage (excitotoxicity) in ischemic stroke but the detailed mechanisms are not fully elucidated. Brain ischemia is also characterized by an impairment of the activity of the proteasome, one of the major proteolytic systems in neurons. We found that excitotoxic stimulation with glutamate rapidly decreases ATP levels and the proteasome activity, and induces the disassembly of the 26S proteasome in cultured rat hippocampal neurons. Downregulation of the proteasome activity, leading to an accumulation of ubiquitinated proteins, was mediated by calcium entry through NMDA receptors and was only observed in the nuclear fraction. Furthermore, excitotoxicity-induced proteasome inhibition was partially sensitive to cathepsin-L inhibition and was specifically induced by activation of extrasynaptic NMDA receptors. Oxygen and glucose deprivation induced neuronal death and downregulated the activity of the proteasome by a mechanism dependent on the activation of NMDA receptors. Since deubiquitinating enzymes may regulate proteins half-life by counteracting ubiquitination, we also analyzed how their activity is regulated under excitotoxic conditions. Glutamate stimulation decreased the total deubiquitinase activity in hippocampal neurons, but was without effect on the activity of Uch-L1, showing that not all deubiquitinases are affected. These results indicate that excitotoxic stimulation with glutamate has multiple effects on the ubiquitin–proteasome system which may contribute to the demise process in brain ischemia and in other neurological disorders.



## 1. Introduction

The ubiquitin–proteasome system (UPS) is the major intracellular machinery for protein degradation. Proteins targeted to be degraded by the proteasome are first conjugated by covalent attachment of ubiquitin chains through the concerted action of a network of enzymes. Firstly, ubiquitin is conjugated to an ubiquitin-activating enzyme, E1, followed by transfer to an ubiquitin conjugating enzyme, E2, and to the ubiquitin ligase, E3, that covalently attaches ubiquitin to a lysine residue on target proteins [1,2]. Poly-ubiquitinated substrates with four or more ubiquitin moieties linked via Lys48 are usually targeted for degradation by the 26S proteasome [1].

The 26S proteasome is the active form of a multisubunit protease complex consisting of a proteolytically active 20S core particle capped at one or both ends by a 19S regulatory particle [3,4]. The 20S proteasome is made up of four stacked rings, two outer α-rings and two inner β-rings. Each ring is comprised of seven different subunits of the α-type and β-type, respectively. Cleavage of peptide bounds is performed in the center catalytic chamber of the 20S core particle by β5, β2 or β1 subunits, which account for the chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome, respectively [5]. The 19S regulatory particle is composed of approximately 20 different subunits which are distributed into two subcomplexes, the lid and the base [3]. The main function of the lid is to capture the ubiquitinated substrates and deubiquitinate them [6,7], while the base complex is responsible for substrate unfolding and opening the gate of the α-ring [3]. Some of the subunits composing the base subcomplex are ATPases, accounting for ATP consumption during proteasome activity.

In the nervous system the UPS plays a role in different neuropathological conditions, including brain ischemia [8]. Transient focal [9] and global [10,11] brain ischemia induces the accumulation of high-molecular weight ubiquitin-conjugated proteins, which were found in clusters with intracellular vesicles in the soma and dendrites, in the nuclear membrane [9,10], and in postsynaptic densities [11]. Furthermore, transient global brain ischemia impairs 26S proteasome

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function by promoting proteasome disassembly, both in rats [12] and gerbils [13]. However, while the 26S proteasome activity recovers in many regions after reperfusion, in more vulnerable areas, such as CA1 region of the hippocampus, the 19S and 20S proteasomes do not fully reassociate [13]. Recent studies showed that after an episode of global brain ischemia, proteasomes are disassembled (partially because of ATP depletion) and some are trapped in ubiquitinated-protein aggregates, and thus fail to function normally [12]. However, proteasome inhibitors promote neuronal survival after focal cerebral ischemia-reperfusion injury, possibly through modulation of the inflammatory response [14].

Brain damage after ischemic stroke and in other neurological disorders is partially due to an extracellular accumulation of glutamate with consequent overactivation of glutamate receptors, leading to an $[Ca^{2+}]_i$ overload and neuronal death (excitotoxicity) [15]. The toxic effects of glutamate are partially mediated by the $Ca^{2+}$-dependent activation of calpains [16]. However, it is not known whether excitotoxic stimulation with glutamate contributes to the deregulation of the UPS. In this work, we show that excitotoxic stimulation of cultured hippocampal neurons downregulates the proteasome and deubiquitinase activities.

## 2. Materials and methods

### 2.1. Hippocampal cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min, 37 °C; GIBCO Invitrogen) in $Ca^{2+}$- and $Mg^{2+}$-free Hank's balanced salt solution (HBSS; 5.36 mM KCl, 0.44 mM $KH_2PO_4$, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4 \cdot 2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (GIBCO Invitrogen), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO Invitrogen), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The cells were dissociated in this solution and were then plated in 6-well plates ($90.0 \times 10^3$ cells/$cm^2$), coated with poly-D-lysine (0.1 mg/ml), or on poly-D-lysine coated glass coverslips, at a density of $80.0 \times 10^3$ cells/$cm^2$. The cultures were maintained in a humidified incubator of 5% $CO_2$/95% air, at 37 °C, for 7 days or 14 days. Hippocampal neurons were then stimulated with Neurobasal medium supplemented with 125 μM glutamate for 20 min, and were allowed to recover in the original cultured medium for the indicated periods of time. When appropriate the cells were pre-incubated with 10 μM of the synthetic penta-peptide LSEAL (kind gift from R. Guttmann, University of Kentucky, Lexington), 50 μM carbobenzoxy-valinyl-phenylalaninal (MDL 28170; Calbiochem), 50 μM Z-Val-Ala-Asp (OMe)-fluoromethylketone (Z-VAD-fmk; Biomol International), 10 μM Z-FY(*t*-Bu)-DMK (DMK; Calbiochem) or with 50 μM [L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA-074Me; Calbiochem), as indicated in the figure captions. All compounds were also present during the stimulation and recovery periods.

### 2.2. Stimulation with AMPA and NMDA, and KCl depolarization

Cultured hippocampal neurons (7 DIV) were independently stimulated with 100 μM AMPA or 100 μM NMDA (Sigma) together with 10 μM glycine, in basal saline solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 6 mM glucose, and 10 mM HEPES), for 20 min, and were then allowed to recover in the original culture medium for 4 h. Stimulation with NMDA was performed in the absence of $Mg^{2+}$ and in the presence of glycine to ensure a maximal neuronal response. Cell extracts were then prepared and processed for proteasome activity assay. To test the effect of KCl depolarization, neurons were pre-incubated with 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris) and 1 μM (5*R*,10*S*)-(−)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801; Tocris), for 5 min, and then stimulated with 30 mM KCl for 20 min, also in the presence of the antagonists. Cells were allowed to recover for 4 h before preparation of the extracts used to measure the proteasome activity. To study the effect of extracellular calcium concentration on the response to NMDA receptor stimulation, 7 DIV cultured hippocampal neurons were incubated or not with 100 μM NMDA and 10 μM glycine, for 20 min, in basal saline solution in the presence or absence of calcium, as depicted in the figure captions. After the stimulation, neurons were further incubated in culture conditioned medium for 4 h.

### 2.3. Activation of synaptic and extrasynaptic NMDA receptors

To upregulate neuronal activity with consequent stimulation of synaptic glutamate receptors, hippocampal neurons (14 DIV) were treated with 50 μM bicuculline (Tocris), 2.5 mM 4-aminopyridine (4-AP; Tocris) and 10 μM glycine for 20 min, as previously described [17,18]. Activation of the extrasynaptic pool of NMDA receptors was conducted by stimulating hippocampal neurons (14 DIV) with 50 μM bicuculline, 2.5 mM 4-AP, 10 μM glycine and 10 μM MK-801 for 5 min, to block synaptic receptors, followed by a washing step with a similar solution but in the absence of MK-801, before incubation with 100 μM NMDA for 20 min. Because MK-801 is an open channel blocker and binds only to active NMDA receptors, the NMDA receptors not stimulated by bicuculline-induced firing (those not located at synapses) are not blocked under these conditions. Therefore, bath application of NMDA after washout of MK-801 presumably acts only on the extrasynaptic pool of NMDA receptors. After stimulation of synaptic or non-synaptic NMDA receptors the cultures were allowed to recover for 4 h in culture conditioned medium before preparation of the extracts. The experiments were performed in basal saline solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$ , 2.5 mM $CaCl_2$, 6 mM glucose, and 10 mM HEPES).

### 2.4. Oxygen-glucose deprivation (OGD) assays

Hippocampal neurons (15 DIV) were incubated for 1.5 h in a solution containing 10 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, pH 7.3, and supplemented with 25 mM glucose (sham cells) or with 25 mM sucrose (OGD) at 37 °C. Sham cells were maintained in an incubator with 5% $CO_2$/95% air, whereas the latter group was incubated in an oxygen deprived chamber (5% $CO_2$, 7.5% $H_2$, 87.5% $N_2$; OGD cells) (Thermo Forma Anaerobic System Model 1029). Cells were further incubated in culture conditioned medium for 4 h (for extracts). When appropriate 20 μM NBQX (Tocris) and 100 μM APV (Tocris) were added 25 min before OGD and were also present during and after the insult. For nuclear morphology analysis, hippocampal neurons (15 DIV) were subjected to OGD for 1.5 h followed by 5 h, 7 h or 12 h of post-incubation in culture conditioned medium. When appropriate 20 μM NBQX and/or 100 μM APV were added 25 min before OGD and were also present during and after the insult.

### 2.5. Cell death

Cell death was evaluated on 15 DIV hippocampal neurons subjected to OGD. The nuclear morphology was analyzed after fixation in 4% sucrose/paraformaldehyde, and incubation with the fluorescent dye Hoechst 33342 (1 μg/ml) for 10 min. The coverslips were then mounted in a fluorescent mounting medium (DAKO), and imaging was performed on a Zeiss Axiovert 200 fluorescence microscope. Three coverslips were prepared for each experimental condition, and at least 200 cells were counted in each case.

### 2.6. Neuron transfection with calcium phosphate

Transfection of cultured hippocampal neurons with Ub-G76V-GFP construct [19] (Addgene plasmid 11941) was performed by the calcium phosphate coprecipitation method as described previously, with minor modifications [20]. Briefly, 10–15 μg of plasmid DNA were diluted in 10 mM Tris-HCl and 0.1 mM EDTA (TE, pH 8.0), and mixed with 2.5 M $CaCl_2$. This DNA/TE/calcium mix was added to 10 mM HEPES-buffered saline solution (270 mM NaCl, 10 mM KCl, 1.4 mM $Na_2HPO4$, 11 mM dextrose, 42 mM HEPES), pH 7.2. The precipitates were allowed to form for 30 min, protected from light, at room temperature, with vortex mixing every 5 min, to ensure that the precipitates had similar small sizes. Meanwhile, cultured hippocampal neurons were incubated with cultured conditioned medium with 2 mM kynurenic acid (Sigma). The precipitate was added drop-wise to each well and incubated at 37 °C/5% $CO_2$, for 3 h. The cells were then washed with HCl acidified culture medium containing 2 mM kynurenic acid and returned to the 37 °C/5% $CO_2$ incubator for 20 min. Finally, the medium was replaced with the initial culture-conditioned medium, and the cells were further incubated in a 37 °C/5% $CO_2$ incubator for 48–72 h to allow protein expression. Cell cultures were than stimulated with 125 μM glutamate for 20 min and allowed to recover for 4 h or 8 h.

### 2.7. Preparation of extracts for proteasome activity

Hippocampal neurons were washed twice with ice-cold phosphate-buffered saline buffer (PBS). The cells were then lysed in 1 mM EDTA, 10 mM Tris–HCl pH 7.5, 20% glycerol, 4 mM dithyothreitol (DTT) and 2 mM ATP (100 μl/well). After sonication and centrifugation at 16,100*g* for 10 min at 4 °C, total protein content in the supernatants was quantified using the Bio-Rad Protein assay, and the concentration of the samples were equalized with lysis buffer.

### 2.8. Native gel electrophoresis

Cultured hippocampal neurons (7 DIV) were washed twice with ice-cold PBS and the cells were then lysed in 100 μl of 50 mM Tris–HCl pH 7.5, 10% glycerol, 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP. The extracts were then centrifuged at 16,100*g* for 10 min at 4 °C, and the protein content in the supernatants was quantified using the Bio-Rad Protein Assay. The protein concentration in the samples was equalized with lysis buffer before separation in 4% polyacrylamide native gels, at 90 V for 4 h (4 °C). For staining purposes, the gels were incubated with 15 ml of 20 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 1 mM ATP and 0.1 mM Suc-LLVY-AMC, for 20 min at 37 °C without agitation, and then analyzed using the EXQuest™ Spot Cutter (Bio-Rad) via a CCD camera in excitation UV mode. The proteins in the gel were then electrotransferred to polyvinylidene (PVDF) membranes and immunoblotted using an antibody against Psma2 (1:1500, Cell Signaling) and Rpt6 (1:1500, Enzo Life Sciences).

### 2.9. Nuclear and cytoplasmic fractionation

Cultured hippocampal neurons (7 DIV) were washed twice with ice-cold PBS and then lysed with 200 μl of buffer 1 containing 10 mM HEPES, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 1 mM EGTA pH 7.5, 4 mM DTT, 2 mM ATP. After incubation for 30 min at 4 °C with agitation, the extracts were centrifuged at 2,000*g* for 10 min. The supernatant (cytoplasmic fraction) was collected and the pellets, containing the nuclear fraction, were resuspended in 70 μl of buffer 2 containing 25 mM HEPES, 300 mM NaCl, 5 mM $MgCl_2$, 20 % Glicerol, pH 7,5, 4 mM DTT, 2 mM ATP. This suspension was then frozen at −80 °C and after thawing it was incubated for 1 h at 4 °C. After centrifugation at 12,000*g* for 20 min at 4 °C, the supernatant (nuclear fraction) was collected and protein quantification was performed using the bicinchoninic acid (BCA) protein assay.

### 2.10. Proteasomal peptidase activity assays

Peptidase activities of the proteasome were assayed by monitoring the production of 7-amino-4-methylcoumarin (AMC) from fluorogenic peptides: Suc-LLVY-AMC (for chymotrypsin-like activity), Z-LLE-AMC (for caspase-like activity) and Boc-LSTR-AMC (for trypsin-like activity; Peptide Institute, Inc). Samples (20 μg for chymotrypsin- and caspase-like activities; 30 μg for trypsin-like activity) were incubated with the fluorogenic substrates, 25 μM Suc-LLVY-AMC, 200 μM Z-LLE-AMC, or 50 μM Boc-LSTR-AMC, in 25 mM Tris–HCl (pH 8.0) and 0.5 mM EDTA buffer, in a final volume of 100 μl. The release of fluorescent AMC was measured at 37 °C using a microplate reader SPECTRAmax Gemini EM (Molecular Devices) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm, for 60 min, at 5 min intervals. All the experiments were performed in the presence of 2 mM ATP. Specific activity was determined by subtracting the activity measured in the presence of 10 μM MG132 (Calbiochem), a proteasome inhibitor.

### 2.11. Western blotting

Hippocampal neurons were washed twice with ice-cold PBS buffer and lysed with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5), supplemented with 1 mM DTT, 50 mM NaF, 1.5 mM sodium orthovanadate and the cocktail of protease inhibitors (0.1 mM PMSF, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin; Sigma-Aldrich Química). After centrifugation at 16,100*g* for 10 min, protein in the supernatants was quantified using the BCA protein assay, and the samples were diluted with a 2× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue).

Protein samples were separated by SDS–PAGE, in 10% polyacrylamide gels, transferred to PVDF membranes (Millipore), and immunoblotted. Blots were incubated with primary antibodies (overnight at 4 °C), washed and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20,000 dilution; 1 h at room temperature). Alkaline phosphatase activity was visualized by ECF on the Storm 860 Gel and Blot Imaging System (GE Healthcare). The following primary antibodies were used: anti-mono and polyubiquitinylated conjugates (1:1000, DakoCytomation), anti-GFP (1:1000, MBL International), anti-Psma2 (1:1500, Cell Signaling), anti-Rpt6 (1:1500, Enzo Life Sciences), anti-Psmc3 (1:1500, Abcam), and anti-Uch-L1 (1:1500, Abcam). Anti-β-Actin (1:6250, Sigma), anti-β-tubulin (1:300000, Sigma), anti-GAPDH (1:5000, Abcam) and anti-histone 3 (1:1000, Cell Signaling) antibodies were used as loading controls.

### 2.12. Measurement of ATP

Cells were washed with ice-cold PBS, scraped, and extracted with 0.6 M perchloric acid, supplemented with 25 mM EDTA-$Na^+$, and centrifuged at 20,800*g* for 5 min at 4 °C. The resulting pellet was solubilized with 1 M NaOH and further analyzed for total protein content by the Bio-Rad Protein assay. After neutralization of the supernatant with 3 M KOH/1.5 M Tris, samples were centrifuged at 20,800*g* for 5 min at 4 °C. The resulting supernatants were assayed for ATP determination by separation in a reverse-phase high-performance liquid chromatography (HPLC), with detection at 254 nm, as described previously [21]. Peak identity was determined by the retention time compared with standards and the amount of ATP was determined by using a concentration standard curve. The

results were expressed in pmol of ATP per mg protein and calculated in percentage of the control.

### 2.13. Total RNA isolation, RNA quality and RNA concentration

Total RNA from 7 DIV cultured hippocampal neurons was extracted with TRIzol (Invitrogen), following the manufacturer's specifications. The full content of a 6-well cluster plate, with a density of $91.6\times10^3$ cells/cm$^2$, was collected for each experimental condition. After the addition of chloroform and phase separation, the RNA was precipitated by the addition of isopropanol. The precipitated RNA was washed once with 75% ethanol, centrifuged, air-dried and resuspended in 60 μl of RNase-free water (GIBCO Invitrogen). The whole procedure was performed at 4 °C.

RNA quality and integrity were assessed using the Experion automated gel-electrophoresis system (Bio-Rad). A virtual gel was created for each sample, allowing the detection of degradation of the reference markers, RNA 18S and 28S. Samples showing RNA degradation or contamination by DNA were discarded. RNA concentration was determined using the fluorescent dye Ribogreen (Invitrogen-Molecular Probes). The samples were aliquoted and stored at −80 °C until further use.

### 2.14. Reverse transcription reaction

For first strand cDNA synthesis 1 μg of total RNA was mixed with Random Hexamer Primer p(dN)$_6$ followed by 10 min denaturation at 65 °C to ensure loss of secondary structures that may interfere with the annealing step. The samples were chilled on ice, and the template-primer mix was then supplemented with Reaction Buffer (50 mM Tris/HCl, 30 mM KCl, 8 mM $MgCl_2$, pH 8.5), Protector RNase Inhibitor (20U), dNTPs (1 mM each) and finally AMV Reverse Transcriptase (10 U; Roche), in a 20 μl final volume. The reaction was performed at 25 °C for 10 min, followed by 30 min at 55 °C, for primer annealing to the template and cDNA synthesis, respectively. The Reverse Transcriptase was then denatured during 5 min at 85 °C, and the samples were then cooled to 4 °C for 5 min, and finally stored at −80 °C until further use.

### 2.15. Primer design

Primers for real-time PCR were designed using the "Beacon Designer 7" software (Premier Biosoft International), and the following considerations were taken: (1) GC content about 50%; (2) annealing temperature ($T_a$) between $55\pm5$ °C; (3) secondary structures and primer-dimers were avoided; (4) primer length between 18 and 24 bp; (5) final product length between 100 and 200 bp.

### 2.16. Real-time PCR

For gene expression analysis 2 μl of 1:100 diluted cDNA was added to 10 μl 2× SYBR Green Master Mix (Bio-Rad) and the final concentration of each primer was 250 nM in 20 μl total volume. The thermocycling reaction was initiated with activation of the Taq DNA polymerase by heating at 95 °C during 30 s, followed by 45 cycles of a 10 s denaturation step at 95 °C, a 30 s annealing step at the optimal annealing temperature for each set of primers, and a 30 s elongation step at 72 °C. The fluorescence was measured after the extension step, using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). After the thermocycling reaction the melting step was performed with slow heating, starting at 55 °C and with a rate of 0.5 °C per 10 s, up to 95 °C, with continuous measurement of fluorescence, allowing detection of possible non-specific products. The assay included a non-template control and a standard curve (in 10-fold steps) of cDNA for assessing the efficiency of each set of primers. All reactions were run in duplicate to reduce confounding variance.

The threshold cycle ($C_t$) represents the detectable fluorescence signal above background resulting from the accumulation of amplified product, and is a proportional measure of the starting target sequence concentration. $C_t$ was measured in the exponential phase and, therefore, was not affected by possible limiting components in the reaction. For every run performed $C_t$ was set at the same fluorescence value. Data analysis was performed using the GenEx (MultiD Analyses) software for real-time PCR expression profiling.

### 2.17. Deubiquitinating enzyme activity assay

Seven DIV hippocampal neurons were washed twice with ice-cold PBS and the cells were then lysed in 20 mM Tris–HCl pH 7.2, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM DTT, 1 μg/ml aprotinin and 1 μg/ml pepstatin (100 μl/well). After sonication and centrifugation at 16,100*g* for 10 min at 4 °C, protein in the supernatants was quantified using the Bio-Rad protein assay, and the concentration of the samples was equalized with lysis buffer. Deubiquitinating enzyme activity was assayed by monitoring the production of AMC from the fluorogenic substrate ubiquitin-AMC (Biomol). One microgram of sample was incubated with 300 nM Ub-AMC, 50 mM Tris–HCl pH 7.5, 1 mM DTT, 10 μg/ml ovalbumin, in a final volume of 100 μl. The release of fluorescent AMC was measured at 37 °C using a microplate reader SPECTRAmax Gemini EM (Molecular Devices) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm, for 20 min, at 1 min intervals.

### 2.18. Active deubiquitinating enzymes

Hippocampal neurons were washed twice with ice-cold PBS. The cells were then lysed in 50 mM Tris–HCl pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP. The extracts were then centrifuged at 16,100*g* for 10 min at 4 °C and the protein in the supernatants was quantified using the Bio-Rad protein assay. The concentration of the samples was equalized with lysis buffer. To assess the active DUBs, 25 μg of protein was incubated with 0.9 μl of DUB active site directed irreversible inhibitor HA-Ub-VS (0.5 μg/μl, Boston Biochem) for 10 min at 30 °C. These samples were then denaturated with 2× concentrated denaturating buffer at 95 °C, for 5 min, and then subjected to Western blot, using the antibody anti-Uch-L1.

### 2.19. Statistical analysis

Statistical analysis was performed using one-way ANOVA analysis of variance followed by the Dunnett's or Bonferroni test, or using the Student's *t* test, as indicated in the figure captions.

## 3. Results

### 3.1. Effect of glutamate-induced excitotoxicity on proteasome activity

To evaluate the effect of excitotoxic stimulation of cultured hippocampal neurons on the proteasome activity, the cells were stimulated with 125 μM glutamate for 20 min and further incubated in culture conditioned medium for different periods of time. Under these conditions, glutamate evokes 40–50% of apoptotic like cell death [22]. Chymotrypsin-, trypsin- and caspase-like activities of the proteasome were assessed using the fluorogenic substrates Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-AMC, respectively. Glutamate stimulation down-regulated the activities of the proteasome in a time-dependent manner, and maximal effects were observed 4 h after the insult. Under the latter conditions the three proteasome activities were decreased by about 50% (Fig. 1A–C). This effect was transient and at longer incubation periods after the excitotoxic insult there was an increase in proteasome activity, which however did not reach the control levels. These results indicate that the insult with glutamate does not cause an

irreversible impairment of the proteasome activities. Excitotoxic stimulation with glutamate also leads to the accumulation of the chimeric protein Ub-G76V-GFP, a fluorescent protein tagged for proteasomal degradation [19], at 4 h or 8 h after the insult (Fig. 1D). These results further indicate that excitotoxic stimulation downregulates the proteasome activity.

To further understand the mechanism underlying the fast impairment of the proteasome activity under excitotoxic conditions we tested whether glutamate stimulation disrupts the integrity of the 26S proteasome complex, thereby affecting its function. This was performed using polyacrylamide-gel electrophoresis under non-denaturating conditions, followed by in-gel assay to evaluate proteasome activity with the fluorogenic substrate Suc-LLVY-AMC. Excitotoxic stimulation with glutamate significantly reduced the chymotrypsin-like activity of the 26S proteasome, as measured 4 h after the insult, and a concomitant increase in the activity of the 20S proteasome was observed (Fig. 2A). Immunoblotting of the native gels with the antibody against the proteasome subunit Psma2, which belongs to the catalytic core, also showed a glutamate-evoked decrease in the amount of protein associated with the 26S proteasome fraction. In agreement with the effect of glutamate on the 20S proteasome activity, excitotoxic stimulation also upregulated the amount of Psma2 present in disassembled proteasomes (Fig. 2B). Moreover, we observed a decrease in Rpt6 protein levels associated with the 26S fraction following the toxic insult with glutamate. As expected, this subunit was not present in the 20S fraction (Fig. 2C), since it belongs to the regulatory particle. Taken together, the results suggest that excitotoxic stimulation with glutamate disassembles the 26S proteasome complex into 20S and 19S subunits, which may significantly reduce protein degradation.

Since the assembly of the 26S proteasome is dependent on the energetic state of the cell, we have also measure the ATP levels after excitotoxic stimulation. We found that 1 h, 4 h or 8 h after glutamate stimulation there is a time-dependent decrease in the ATP content of the cells (Fig. 3). This depletion in the ATP levels is correlated with the observed disassembly of the 26S proteasome.

### 3.2. Effect of excitotoxic stimulation with glutamate on the activity of the proteasome in cytoplasmic and nuclear fractions

The proteasomes are present in the cytoplasm and in the nucleus [23] and, therefore, we investigated whether excitotoxic stimulation differentially regulates the two populations of proteasomes in hippocampal neurons. Measurement of the chymotrypsin-like activity of the proteasome in nuclear and cytoplasmic fractions isolated from hippocampal neurons showed a significant downregulation in the nuclear fraction 4 h after the excitotoxic insult, but no significant change was observed in the cytoplasm under the same conditions (Fig. 4). These results indicate that glutamate-induced excitotoxicity mainly targets the nuclear pool of the proteasome. Interestingly, Western blot experiments showed a significant increase of Psma2 protein levels in the nuclear pool of glutamate stimulated cells when compared with non-stimulated neurons, but no significant effect was observed in the cytoplasmic fraction (Fig. 4C). No changes were observed in the abundance of Rpt6 in the two cellular fractions after glutamate stimulation (Fig. 4D), suggesting that the 19S regulatory particle is the assembling limiting component in the nucleus.

To further understand the effect of glutamate stimulation on the UPS, and because a proteasome failure increases polyubiquitinated protein levels, we evaluated the levels of mono- and polyubiquitin conjugates in nuclear and cytoplasmic fractions of unstimulated and glutamate stimulated cells by Western blot (Fig. 4B). The results showed a significant increase in high molecular weight polyubiquitin

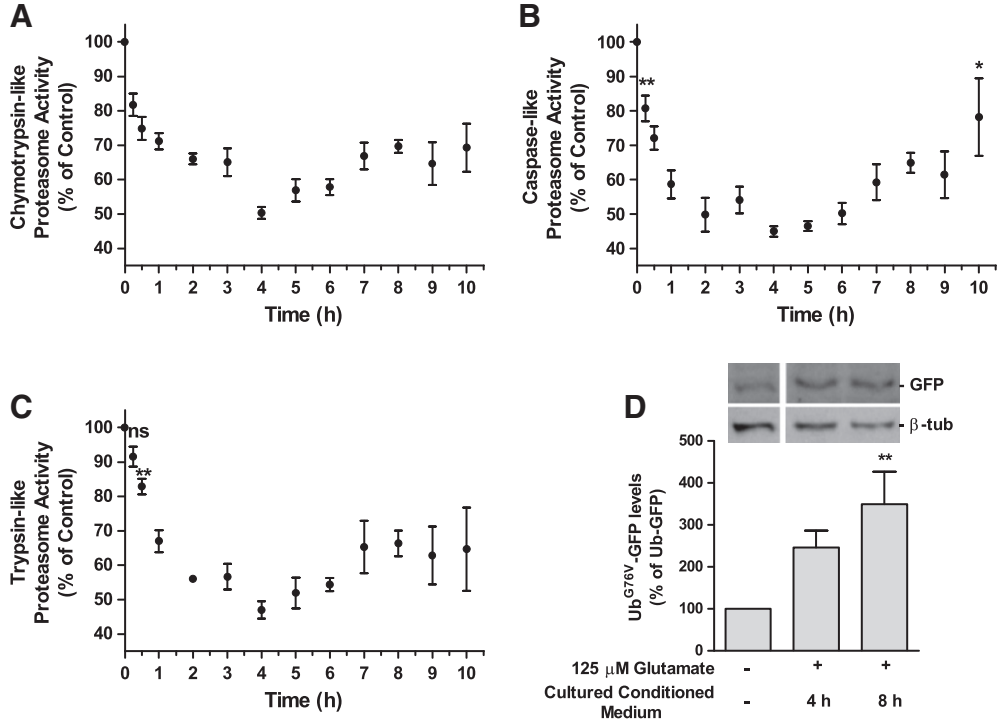


**Fig. 1.** Effect of excitotoxic stimulation with glutamate on the activity of the proteasome in cultured hippocampal neurons. A–C) The cells (7 DIV) were stimulated with 125 μM glutamate, for 20 min, and the activities of the proteasome were measured at the indicated periods of time after the toxic insult. The three different activities, chymotrypsin- (A), caspase- (B) and trypsin-like (C), were measured with the fluorogenic substrates 25 μM Suc-LLVY-AMC, 200 μM Z-LLE-AMC and 50 μM Boc-LRR-AMC, respectively. The control proteasome activity, determined in non-stimulated neurons, was set to 100%. The results are the average ± SEM of 3 to 8 experiments, performed in independent preparations. Statistical analysis was performed by ANOVA, followed by Dunnett's post test, comparing all the conditions with the control. All effects are statistically significant with $^{***}P<0.001$, except where indicated as n.s. ($P>0.05$), $^{*}P<0.05$ and $^{**}P<0.01$. D) Ub-G76V-GFP transfected hippocampal neurons were incubated with 125 μM glutamate for 20 min and further incubated in culture conditioned medium for 4 h or 8 h. The abundance of Ub-(G76V)-GFP was evaluated by Western blot using an anti-GFP antibody. The control Ub-(G76V)-GFP levels, determined in non-stimulated neurons, was set to 100%. The results are the average ± SEM of 3–4 independent experiments performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test ($^{**}P<0.01$).

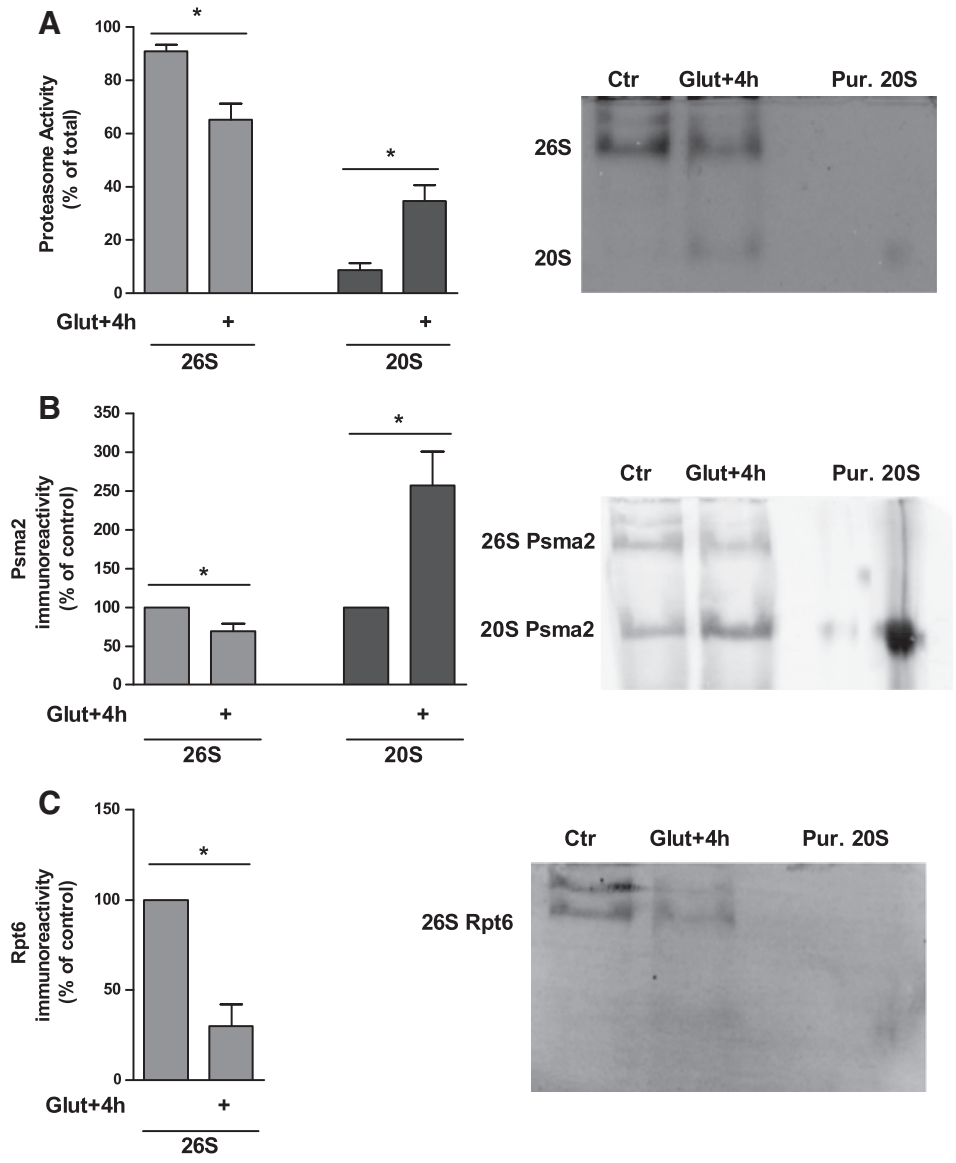


**Fig. 2.** Effect of excitotoxic stimulation with glutamate on proteasome assembly in cultured hippocampal neurons. The cells (7 DIV) were stimulated or not (Ctr) with 125 μM glutamate, for 20 min, and further incubated in culture conditioned medium for 4 h. A) In-gel proteasome activity after native gel electrophoresis, using 0.1 mM Suc-LLVY-AMC. B) Abundance of Psma2 in non-denaturating conditions after immunoblotting the native gel. C) Abundance of Rpt6 in non-denaturating conditions after immunoblotting the native gel. Pur. 20S shows the results with a commercial fraction of purified human erythrocyte 20S proteasome (Biomol), used as a positive control. The two bands observed in all the representative images for the 26S proteasome consist in single- (lower band) or double-capped 26S proteasome (upper band). For data analysis both bands were considered 26S proteasome. Control (unstimulated) proteasome activity was set to 100%. The results are the average ± SEM of 3 experiments, performed in independent preparations. Statistical analysis was performed by Student's *t* test. $^{*}P<0.05$.

conjugates in the nuclear and cytoplasmic fractions following excitotoxic stimulation, with more pronounced effects on the nuclear fraction (Fig. 4B).

### 3.3. Differential effect of glutamate receptors and voltage sensitive calcium channels on the activity of the proteasome

Although it is well established that $Ca^{2+}$-entry through NMDA receptors plays a critical role in excitotoxic cell death [24,25], AMPA receptors also contribute to glutamate-induced neuronal death (e.g. Ref. [26]). Thus, we evaluated the relative role of AMPA and NMDA receptors in the glutamate-induced changes in proteasome activity. Hippocampal neurons were treated with 100 μM AMPA or 100 μM NMDA for 20 min and the proteasome activity was measured 4 h after stimulation. Activation of NMDA receptors significantly decreased chymotrypsin- and caspase-like activities of the proteasome (Fig. 5A–C), in contrast with AMPA receptors, which were without effect on the activity of the proteasome (Fig. 5A–C).

Glutamate stimulation may also induce the influx of $Ca^{2+}$ through voltage-gated $Ca^{2+}$ channels (VGCC). Studies in hippocampal cultures have shown that $Ca^{2+}$ entry mediated by NMDA receptors and by VGCC (stimulated by KCl) activate two distinct signaling pathways which target different enhancer elements [27]. Therefore, we evaluated the effect of KCl depolarization and the consequent activation of VGCC on proteasome activity. Hippocampal neurons were simulated with 30 mM KCl for 20 min, in the presence of 20 μM CNQX and 1.0 μM MK-801, to block AMPA and NMDA receptors, respectively, and the cells were further incubated in culture-conditioned medium for 4 h before measuring proteasome activity. Calcium entry mediated by VGCC did not change proteasome activity (Fig. 5A–C). These results suggest that NMDA receptor activation is the main responsible for the downregulation of proteasome activity.

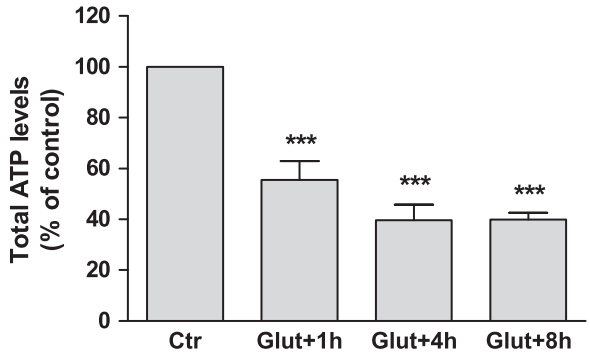


**Fig. 3.** Effect of excitotoxic stimulation with glutamate on ATP levels. Cultured hippocampal neurons (7 DIV) were stimulated or not (Ctr) with 125 μM glutamate, for 20 min, and further incubated in culture conditioned medium for 1 h, 4 h and 8 h. Intracellular ATP was determined by HPLC as described in Materials and methods and the results were calculated as pmol ATP/mg of protein. Data are expressed as the percentage of control (100%) and are mean ± S.E.M. from 6 independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test ($^{***}P<0.001$).

Given the role of $Ca^{2+}$ entry through NMDA receptors in excitotoxic cell death [24,25] we investigated the role of extracellular $Ca^{2+}$ in the NMDA-induced downregulation of the proteasome. The absence of extracellular $Ca^{2+}$ prevented the decrease in the chymotrypsin-like activity of the proteasome induced by NMDA stimulation (Fig. 5D), suggesting that calcium influx through NMDA receptors is coupled to the activation of downstream signaling pathways that downregulate proteasome proteolytic activity.

Proteasome activity was also evaluated after OGD, an *in vitro* model of transient global ischemia [28]. Thus, 1.5 h of OGD followed by 4 h incubation in culture-conditioned medium decreased the proteasome chymotrypsin-like activity in cultured hippocampal neurons (Fig. 5E), and this effect was abrogated by the combined inhibition of NMDA and AMPA receptors, with APV and NBQX, respectively, or in the presence of the NMDA receptor inhibitor tested alone (Fig. 5E). The OGD conditions used induced the death of 14.8%, 30.9% and 30.1% of the neurons, as determined 5 h, 7 h or 14 h after the insult using Hoechst 33342 (Fig. 5F), and these effects were partially inhibited by APV. NBQX together with APV did not further decrease the percentage of cell death when compared with APV alone. Taken together, these results suggest that NMDA receptors play an important role on the regulation of proteasome activity under excitotoxic conditions.

### *3.4. Extrasynaptic NMDA receptors are preferentially coupled to the regulation of the proteasome*

During pathological conditions, massive release of glutamate is expected to activate both synaptic and extrasynaptic ionotropic glutamate receptors. Recent findings show that the location of $Ca^{2+}$ entry into the cells critically influences the fate of neurons [17,18]. Since activation of NMDA receptors specifically decreases proteasome activity (Fig. 5), we examined the differential effect of synaptic and extrasynaptic NMDA receptor activation on proteasome function. Synaptic glutamate release was evoked by application of bicuculline, a blocker of GABA receptors, which removes the inhibitory action of

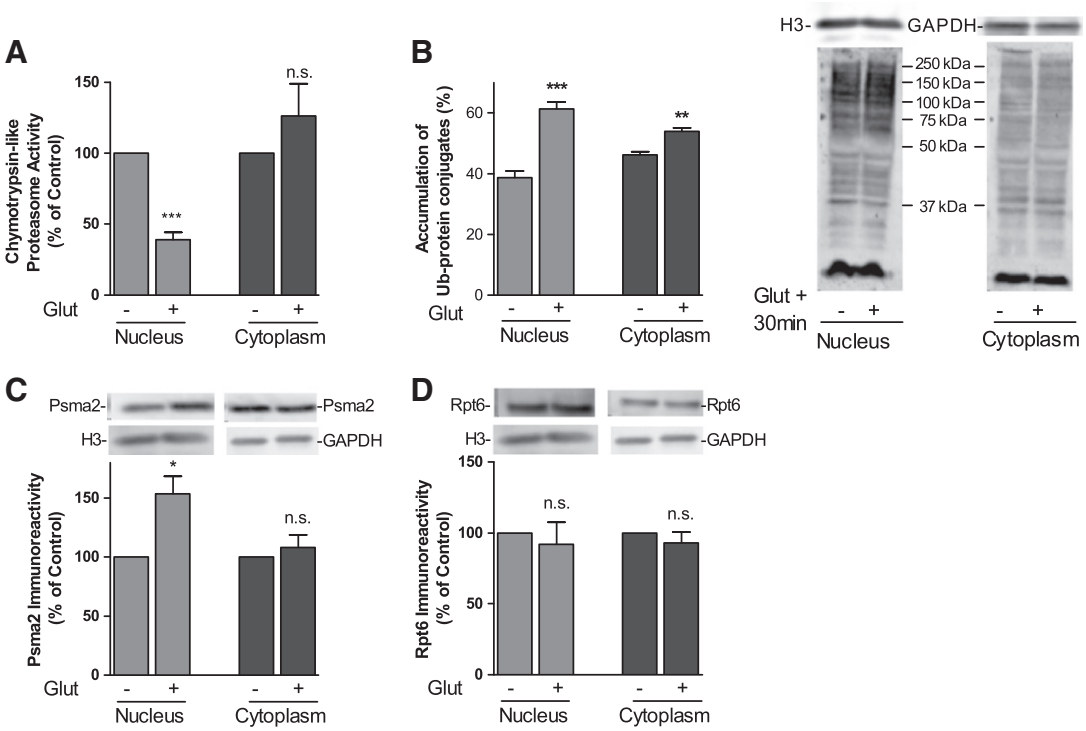


**Fig. 4.** Effect of excitotoxic stimulation with glutamate on the UPS in nuclear and cytoplasmic fractions isolated from cultured hippocampal neurons. Cultured hippocampal neurons (7 DIV) were stimulated or not with 125 μM glutamate, for 20 min, and further incubated in cultured conditioned medium for 30 min (C) or 4 h (A, B) before preparation of the nuclear and cytoplasmic fractions. A) Effect on chymotrypsin-like activity in nuclear and cytoplasmic fractions, as measured with the fluorogenic substrate Suc-LLVY-AMC (25 μM). Control (unstimulated) proteasome activity of nuclear and cytoplasmic fractions was set to 100%. The results are the average ± SEM of 4 to 5 experiments, performed in independent preparations. Statistical analysis was performed by Student's *t* test. $^{***}P<0.001$. B) Levels of mono- and polyubiquitinated conjugates, measured by Western blot using anti-ubiquitin antibody (DakoCytomation), in the nuclear and cytoplasmic fractions of control and glutamate stimulated cells. Only the 50 kDa–250 kDa proteins were quantified and the results are shown on the left panel. Statistical analysis was performed by Student's *t* test. $^{**}P<0.01$, $^{***}P<0.001$. C) Psma2 protein levels in the nuclear and cytoplasmic fraction of hippocampal neurons evaluated by Western blot, using an anti-Psma2 antibody. The results are the average ± SEM of 4 independent experiments. Statistical analysis was performed by Student's *t* test. $^{*}P<0.05$. D) Abundance of Rpt6 in the nuclear and cytoplasmic fractions of hippocampal neurons evaluated by Western blot, using an anti-Rpt6 antibody. The results are the average ± SEM of 4–5 independent experiments, performed in different preparations. Statistical analysis was performed by Student's *t* test.

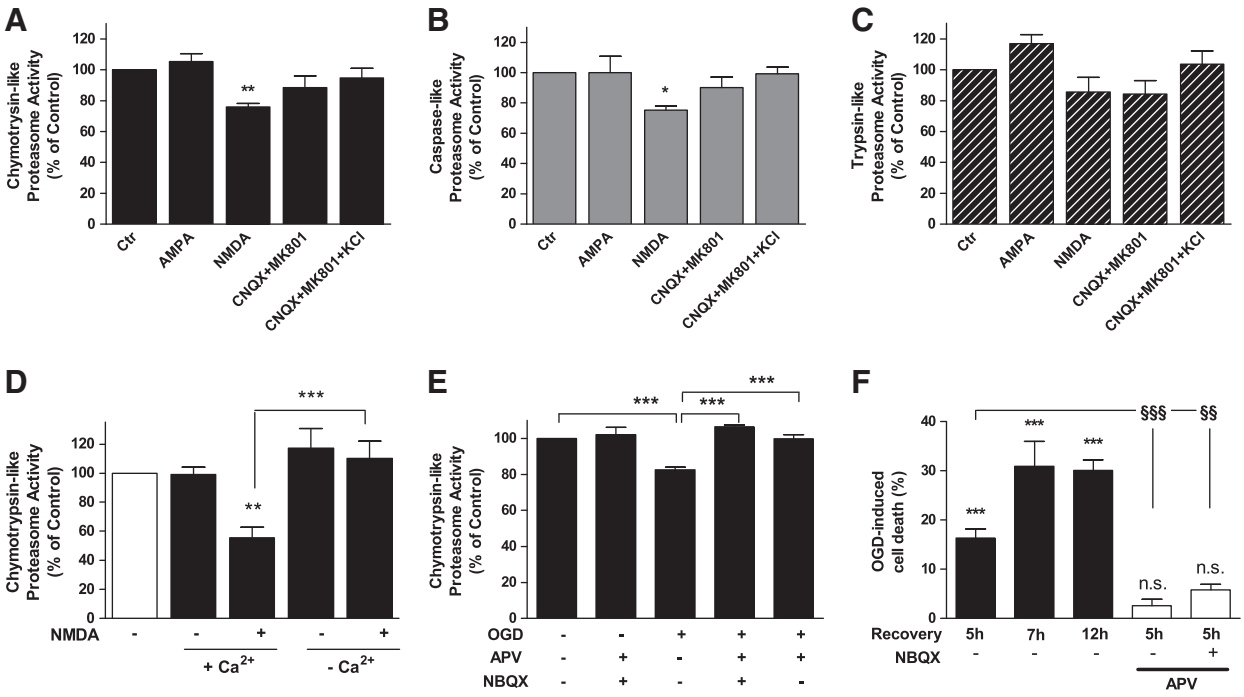


**Fig. 5.** Effect of AMPA, NMDA, and KCl depolarization on proteasome activity in cultured hippocampal neurons. A–C) The cells (7 DIV) were stimulated with 100 μM NMDA in the presence of 10 μM glycine in a $Mg^{2+}$-free $Na^{+}$-salt solution, for 20 min. Stimulation with 100 μM AMPA and depolarization with 30 mM KCl were performed during the same period of time. KCl depolarization was performed in the presence of 20 μM CNQX and 1 μM MK-801. Cells were pre-incubated with the antagonists for 5 min and the inhibitors were also present during KCl-induced depolarization. Proteasome activity was measured 4 h after stimulation, using the following fluorogenic substrates: 25 μM Suc-LLVY-AMC, 200 μM Z-LLE-AMC, and 50 μM Boc-LSTR-AMC to assay (A) chymotrypsin-, (B) caspase-, and (C) trypsin-like activities, respectively. Control (unstimulated) proteasome activity was set to 100%. The results are the average ± SEM of 6 to 11 experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test, comparing all conditions with the control. $^{*}P<0.05$, $^{**}P<0.01$. D) Effect of extracellular calcium on proteasome activity upon NMDA stimulation. Seven DIV cultured hippocampal neurons were stimulated or not for 20 min with 100 μM NMDA and 10 μM glycine, in basal saline solution in the presence or absence of calcium. After the stimulation, neurons were allowed to recover for 4 h in culture conditioned medium. Control (unstimulated cells) proteasome activity was set to 100%. The results are the average ± SEM of 5 different experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test, comparing all conditions with the control, $^{**}P<0.01$, or Bonferroni's multiple comparison test. $^{***}P<0.001$. E) Effect of OGD on the proteasome activity. Fifteen DIV neurons were subjected to OGD for 1.5 h and further incubated in cultured conditioned medium for 4 h. When 100 μM APV and/or 20 μM NBQX were used, the inhibitors were added 25 min prior and during OGD. Proteasome chymotrypsin-like activity was measured using the fluorogenic substrate 25 μM Suc-LLVY-AMC. Proteasome activity of Sham condition was set to 100%. The results are the average of ± SEM of 3 to 7 independent experiments performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Bonferroni's multiple comparison test. $^{***}P<0.001$. F) Effect of OGD on cell death. Fifteen DIV neurons were subjected to OGD for 1.5 h and further incubated in cultured conditioned medium for 5 h, 7 h and 12 h. When 100 μM APV and/or 20 μM NBQX were used, the inhibitors were added 25 min prior and during OGD. After fixation, cell death was determined by fluorescence microscopy with Hoechst 33342. The results are the average of ± SEM of 3 to 6 independent experiments performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test ($^{*}P<0.05$, $^{***}P<0.001$) or by Student's *t* test ($^{§§}P<0.01$).

GABA and thus increases the glutamate-driven activity of the neuronal network, and selective activation of extrasynaptic NMDA receptors was performed as previously described [17,18]. The results show that activation of synaptic receptors does not modulate proteasome activity (Fig. 6). In contrast, selective activation of the extrasynaptic pool of NMDA receptors significantly decreased the chymotrypsin, caspase- and trypsin-like activities of the proteasome (Fig. 6), suggesting that activation of extrasynaptic NMDA receptors and downstream signaling pathways play a key role in the modulation of the proteasome activity. Although $Ca^{2+}$ entry following NMDA

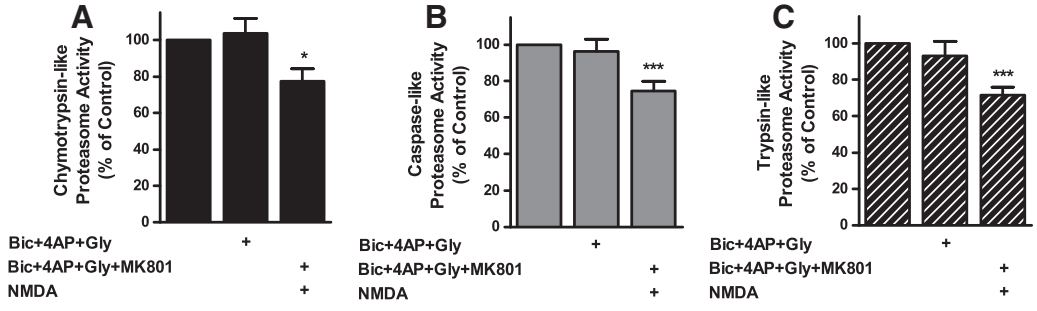


**Fig. 6.** Effect of synaptic and extrasynaptic NMDA receptor stimulation on proteasome activity in cultured hippocampal neurons. To induce synaptic activity neurons (14 DIV) were treated with 50 μM bicuculline, 2.5 mM 4-AP and 10 μM glycine, for 20 min. Selective activation of extrasynaptic NMDA receptors was performed after blocking the synaptic receptors, with 50 μM bicuculline, 2.5 mM 4-AP, 10 μM glycine, and 1 μM MK-801, for 5 min. Extrasynaptic NMDA receptors were then stimulated for 20 min with 100 μM NMDA in the absence of MK-801. Proteasome activity was measured 4 h after stimulation, using fluorogenic substrates: 25 μM Suc-LLVY-AMC, 200 μM Z-LLE-AMC, and 50 μM Boc-LSTR-AMC to assay (A) chymotrypsin-, (B) caspase-, and (C) -trypsin-like activities, respectively. Control (unstimulated) proteasome activity was set to 100%. The results are the average ± SEM of 11 to 13 experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's test, comparing all the conditions with the control. $^{*}P<0.05$, $^{***}P<0.001$.

receptor activation may stimulate several intracellular cascades, inhibition of the proteasome is not a direct effect of $Ca^{2+}$ since the chymotrypsin-like, caspase-like and trypsin-like activities, measured in media with different free calcium concentrations (0–250 μM), were not significantly different (Supplementary Figure 1 ).

### 3.5. Effect of proteases on the proteasome activity after the excitotoxic insult

The intracellular $Ca^{2+}$ overload due to excessive activation of glutamate receptors upregulates several $Ca^{2+}$-dependent proteases, including calpains [29] and caspases [22], which cleave a wide range of substrates, thereby contributing to neurodegeneration. Cysteine proteases belonging to the calpain and caspase families are major players in the apoptotic neuronal death following brain ischemia [29] and excitotoxic insults [22,30]. Calpain inhibition with 10 μM L-SEAL or 50 μM MDL 28170 was without effect on the glutamate-induced decrease in proteasome activity (Fig. 7). Inhibition of caspases with 50 μM Z-VAD-fmk slightly reduced (16.7%) the downregulation of the proteasome chymotrypsin-like activity induced by glutamate stimulation. However, Z-VAD-fmk also increased the basal chymotrypsin-like activity under control conditions (18.1%; Fig. 7A), suggesting that caspases do not contribute to the alteration in proteasome activity under excitotoxic conditions (Fig. 7A–C). The lysosomal cysteine proteases, cathepsin-B and cathepsin-L are also involved in ischemic cerebral damage [31]. Inhibition of cathepsin-L with 10 μM DMK significantly prevented the decrease of proteasome chymotrypsin-like activity induced by glutamate (Fig. 7A), but the cathepsin-B inhibitor 50 μM CA-074-Me was without effect. No effects were observed for the proteasome caspase- and trypsin-like activities (Fig. 7B and C). Taken together, these results suggest that activation of cathepsins-L is a key event in the excitotoxicity-induced decrease of proteasome activity. Western blot and quantitative PCR experiments showed no changes in the protein and expression levels of two subunits of the 20S proteasome (Psma2 and Psmb7) and one subunit of the 19S proteasome (Psmc3) (Fig. 8), ruling out an effect of the proteases on these proteasome components.

### 3.6. Effect of excitotoxic stimulation on deubiquitinating enzyme activity, expression and protein levels

Proteasome-associated deubiquitinating enzymes (DUBs) are present at the 19S subunit and account for the cleavage of the ubiquitin moieties that target a protein for degradation, contributing to the translocation of proteins to the 20S catalytic core. Therefore, changes in their activity as well as in the activity of cytoplasmic DUBs affect protein degradation at the 20S catalytic pore. Thus, using the fluorogenic substrate Ub-AMC, we evaluated the total DUB activity in hippocampal neurons subjected to excitotoxic stimulation with glutamate. The results show a significant decrease in DUB activity starting at 2 h after the toxic insult (Fig. 9A).

DUBs constitute one of the largest classes of human proteases. One class of DUBs (e.g. Usp14) removes proximal ubiquitins from target proteins, whereas other proteases target distal ubiquitins (e.g. ubiquitin C-terminal hydrolase [Uch-L1]) [32]. Thus, we tested whether the Uch-L1 activity was affected by glutamate stimulation, by incubating cellular extracts with the DUB active site directed inhibitor HA-Ub-VS, followed by a Western blot using an antibody against Uch-L1. The inhibitor binds to DUBs active site when the enzymes are active, causing a 10 kDa shift in the enzyme apparent molecular weight (Fig. 9B). No difference was observed in the activity of Uch-L1 after excitotoxic stimulation with glutamate (Fig. 9B), indicating that the decrease in DUB activity observed using the fluorogenic substrate is not a general effect induced by glutamate, but is due to inhibition of specific DUBs. Furthermore, we found no differences in the gene expression levels of *Uch-L1* and *Usp14* at 15 min after excitotoxic stimulation. However, after 4 h of incubation in culture conditioned medium there was a significant decrease on the mRNA levels of *Uch-L1* and *Usp14* (Fig. 9C and D), although no changes were observed in the abundance of Uch-L1 protein (Fig. 9C). These results point to a selective effect of excitotoxic stimulation on the activity of specific DUBs, while others are not affected.

## 4. Discussion

The UPS is the major non-lysosomal pathway for intracellular protein degradation, playing a key role in controlling protein quality by removing damaged, oxidized and/or misfolded proteins. Impairment of UPS-mediated proteolysis and consequent accumulation of ubiquitinated proteins leads to neuronal dysfunction and is a possible cause of neurodegeneration seen in many diseases of the nervous system [33]. In this work we show that transient OGD and excitotoxic exposure to glutamate downregulates the proteasome activity in cultured hippocampal neurons. The observed downregulation of the proteasome activity in OGD and under excitotoxic conditions is correlated with the impairment of proteasome function observed in the frontal cortex and hippocampus after transient global forebrain ischemia [13], and suggests that overactivation of glutamate receptors may mediate the downregulation of the UPS.

The downregulation of proteasome activity was transient and at longer periods after excitotoxic stimulation there was a recovery, although it did not reach the control levels. This recovery may arise, at least in part, from an increase of proteasomal activity in hippocampal neurons that remained alive and may allow overcoming the initial cellular changes induced by the excitotoxic insult. The transient effects on the proteasome activity are correlated with the fast but transient loss of mitochondrial membrane potential and decrease in ATP levels observed in cerebellar granule neurons subjected to excitotoxic stimulation with glutamate, which is followed by apoptotic-like cell

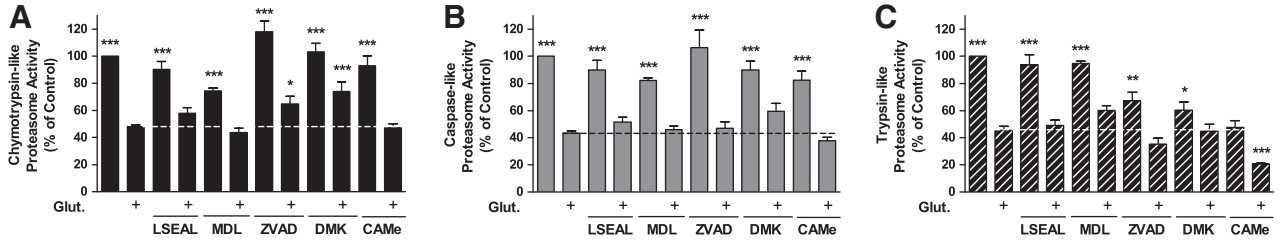


**Fig. 7.** Effect of protease inhibitors on the proteasome activity in cultured hippocampal neurons after excitotoxic stimulation. Seven DIV hippocampal neurons were stimulated with 125 μM glutamate for 20 min, and the activities were measured 4 h after the toxic insult. Proteasome activity was measured using the fluorogenic substrates 25 μM Suc-LLVY-AMC, 200 μM Z-LLE-AMC, and 50 μM Boc-LSTR-AMC to assay (A) chymotrypsin-, (B) caspase-, and (C) trypsin-like activities, respectively. The inhibitors used were 10 μM LSEAL and 50 μM MDL 28170, for calpain inhibition, 50 μM Z-VAD-fmk for caspase inhibition, and 50 μM CA-074-Me and 10 μM DMK to inhibit cathepsin-B and -L, respectively. Cells were pre-incubated with the inhibitors for 2 h before glutamate stimulation and the inhibitors were also present during glutamate stimulation. Control (unstimulated) proteasome activity was set to 100%. The results are the average ± SEM of 4 to 8 experiments, performed in independent preparations. Statistical analysis was performed by one-way ANOVA, followed by the Bonferroni's multiple comparison test, comparing the condition with glutamate alone with all the other conditions. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

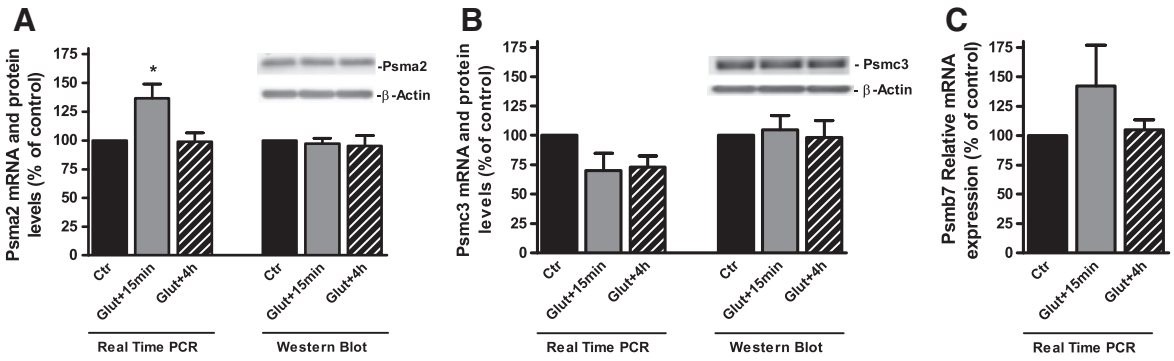


**Fig. 8.** Effect of glutamate excitotoxicity on the expression and protein abundance of the Psma2, Psmc3 and Psmb7 proteasome subunits. Seven DIV hippocampal neurons were stimulated with 125 μM glutamate for 20 min, and the expression of *Psma2* (A), *Psmc3* (B) and *Psmb7* (C) genes was determined after 15 min or 4 h of incubation in culture conditioned medium. Normalization was performed against the *Gapdh* control gene and taking in account the $C_t$ values of the real-time PCR assays for each gene. Control mRNA levels, measured in unstimulated cells, were set to 100%. The mRNA expression results are the average ± SEM of 5–6 independent transcription reactions, performed in independent preparations. $^{*}P<0.05$ as determined by Student's *t*-test performed on log-transformed expression data. Psma2 (A) and Psmc3 (B) protein levels were determined by Western blot in extracts prepared from cells subjected to the same experimental conditions as for the real-time PCR assay. β-Actin was used as loading control in the Western blot experiments, and the results are expressed as a ratio with β-actin protein levels. The control ratio for each protein was set to 100%. The Western blot results are the average ± SEM of 5–6 independent experiments, performed in independent preparations. Statistical analysis was performed by one way ANOVA, followed by the Dunnett's test, comparing all the conditions with control. $^{*}P<0.05$.

death [34]. Furthermore, transient excitotoxic stimulation with glutamate was also shown to induce a reversible depolarization of the plasma membrane followed by hyperpolarization of the mitochondrial membrane and increase in ATP levels before a delayed apoptotic-like cell death occurs [35]. Taken together, these results suggest that the initial insult resulting from excitotoxic stimulation activates regulatory mechanisms which may contribute to the observed recovery of the proteasome activity. These metabolic responses may prevent a rapid cell death, such as the necrotic neuronal death observed upon sustained activation of glutamate receptors, and may contribute to a delayed demise of the cells.

The 26S proteasome is formed by assembly of 20S and 19S subcomplexes in an ATP-dependent manner [4,36] and, therefore, proteasome integrity is highly dependent on the energetic state of

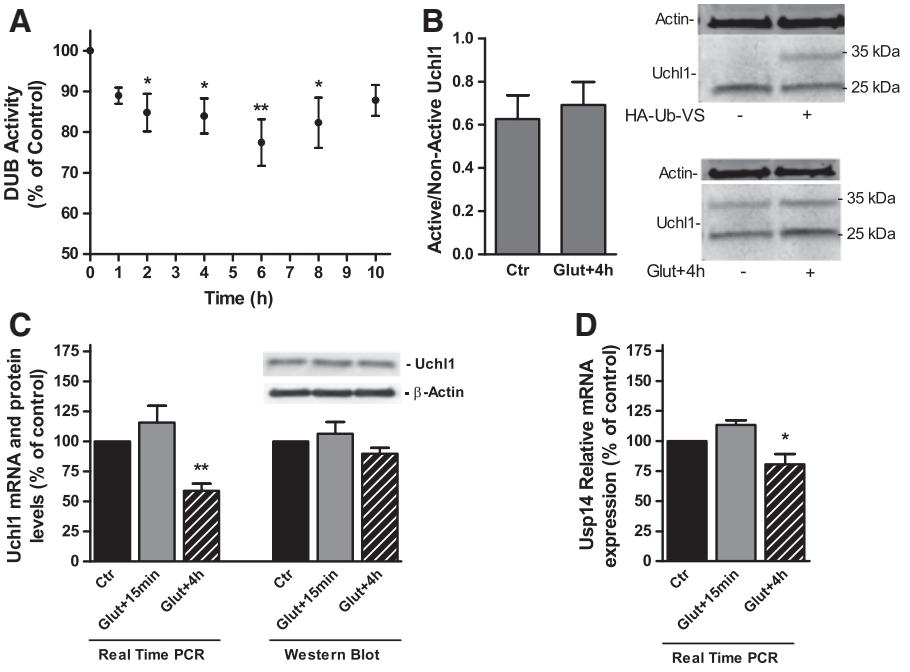


**Fig. 9.** Effect of glutamate excitotoxicity on the activity, expression and protein abundance of DUBs in cultured hippocampal neurons. Seven DIV hippocampal neurons were stimulated with 125 μM glutamate for 20 min, and the cells were further incubated in culture conditioned medium for the indicated periods of time. A) The DUBs overall activity was measured at 1 h to 10 h after the excitotoxic insult, using the fluorogenic substrate Ub-AMC (300 nM). Control (unstimulated cells) DUB activity was set to 100%. The results are the average ± SEM of 5 to 8 experiments, performed in independent preparations. B) The ratio of active versus non-active Uch-L1 was measured by Western blot using an antibody against Uch-L1, using extracts incubated with the inhibitor HA-Ub-VS directed against the active site of DUBs. The results are the average ± SEM of 5 independent experiments. C and D) Expression of *Uch-L1* (C) and *Usp14* (D) in hippocampal neurons 15 min or 4 h after excitotoxic stimulation. Normalization was performed against the *Gapdh* control gene and taking in account the $C_t$ values of the real-time PCR assays for each gene. Control mRNA levels, determined in non-stimulated cells, were set to 100%. The mRNA expression results are the average ± SEM of 5–6 independent transcription reactions, performed in independent preparations. $^{*}P<0.05$, $^{**}P<0.01$ as determined by Student's *t*-test performed on log-transformed expression data. Uch-L1 protein levels (C) were determined by Western blot in cell extracts prepared from hippocampal neurons subjected to the indicated experimental conditions. β-Actin was used as loading control, and the results are expressed as a ratio with β-actin protein levels. The control ratio of a protein was set to 100%. The Western blot results are the average ± SEM of 5–6 independent experiments, performed in independent preparations. Statistical analysis was performed by one way ANOVA, followed by the Dunnett's test, comparing all the conditions with control. $^{*}P<0.05$.

the cells. The in-gel assay of the proteasome activity showed a significant disassembly of the 26S proteasome under excitotoxic conditions, with a consequent upregulation of the 20S form, which is inactive in the degradation of ubiquitinated proteins. The disassembly of the 26S proteasome following excitotoxic stimulation may be due, at least in part, to the observed decrease in the ATP content, which is also observed within a few minutes after brain ischemia [37]. A disassembly and aggregation of the proteasome was also reported after global brain ischemia with the two-vessel occlusion model in rats [12]. In contrast with the results obtained in this work, non-toxic stimulation of NMDA receptors reduced poly-ubiquitination species, possibly by affecting specific E3 ligases [38]. An accumulation of ubiquitin-conjugated proteins was observed in the nucleus and cytoplasm, although with a larger increase in the nuclear fraction where a downregulation of the chymotrypsin-like activity of the proteasome was observed. Increased levels of poly-ubiquitinated species may be due to a decrease in ubiquitin removal (by DUBs) and/or due to increased ubiquitination. The differences observed for the proteasome activity in the nuclear and cytoplasmic fractions after the same excitotoxic stimulus may arise by differential regulation of intracellular signaling molecules in the two intracellular compartments.

The glutamate-evoked downregulation of proteasome activity was selectively induced by non-synaptic NMDA receptors. Activation of synaptic NMDA receptors did not affect the proteasome activities, similarly to the effect of AMPA receptor stimulation or KCl depolarization. Interestingly, previous work have shown that calcium influx evoked by activation of synaptic NMDA receptors is not toxic whereas similar $Ca^{2+}$ loads, resulting from stimulation of extrasynaptic NMDA receptors, inactivates the CREB pro-survival program and promotes breakdown of mitochondrial membrane potential and cell death [17,18]. Moreover, $Ca^{2+}$ influx through NMDA receptor channels was shown to be preferentially associated with neuronal death when compared with $Ca^{2+}$-permeable AMPA receptors in retinal neurons [39], and for equivalent $Ca^{2+}$ loads KCl-depolarization and activation of L-type $Ca^{2+}$ channels was shown to be much less efficient than NMDA receptors in inducing cell death in hippocampal neurons [40]. Interestingly, NMDA receptors also contributed to the downregulation of the proteasome chymotrypsin-like activity in hippocampal neurons subjected to transient OGD, and the activation of ionotropic glutamate receptors contributed to neuronal death under the experimental conditions used. A previous study showed that OGD also impairs proteasome activity in cortical neurons by disrupting $Na^{+}$ and $Ca^{2+}$ homeostasis [28].

It is not clear at this point how activation of non-synaptic NMDA receptors is coupled to the deregulation of the nuclear proteasome activity and why the cytoplasmic proteasome is not significantly affected. However, this work suggests that $Ca^{2+}$ entry through NMDA receptor channels may play a key role in the NMDA-induced proteasome dowregulation. Downregulation of the proteasome activity under excitotoxic conditions is not a direct consequence of the $[Ca^{2+}]_i$ overload, but might be due to the activation of specific intracellular signaling mechanisms since a similar activity of the proteasome was observed in media with different free $Ca^{2+}$ concentrations (Supplementary Figure 1). Although $Ca^{2+}$ influx through VGCC induces proteasome activity in hippocampal neurons [41], in the present work the effect of glutamate on UPS was probably independent of calcium variations. Additionally, our results suggest the involvement of cathepsins in proteasome deregulation since inhibitors of these proteases partially prevented the downregulation of the chymotrypsin-like activity of the proteasome. Accordingly, lysosomes, the reservoirs of cathepsins, were shown to be involved in the degradation of rat liver proteasomes [42]. Furthermore, cathepsins-B and -L were shown to play a role in focal ischemia-induced cell death [31]. In contrast, the pan-caspase inhibitor did not affect significantly the down-regulation of the proteasome activity, in agreement with the minor role played by caspases in excitotoxic cell death [43]. Post-translational modifications of the 26S proteasome components, including phosphorylation, may also contribute to the regulation of proteasome activity under excitotoxic conditions. Accordingly, auto-phosphorylation of the Rpt6-subunit in the dissociated 19S complex may elicit 26S assembly [44], and several 20S subunits are known to be phosphorylated [45].

Excitotoxic stimulation of hippocampal neurons upregulated Psma2 protein levels in the nuclear compartment, which obviously cannot account for the observed downregulation of the proteasome activity. These results may reflect an increase of the nuclear import of Psma2 after a toxic insult or it may be due to an upregulation in Psma2 synthesis followed by targeting to the nucleus. Under the same conditions no effect was observed on the cytoplasmic or total Psma2 levels, which may be due to the relative abundance of proteasomes in the cytoplasmic and nuclear compartments. NMDA receptor stimulation was also shown to change the distribution of the proteasomes within the dendrites in cultured hippocampal neurons [46].

Free $Zn^{2+}$ accumulates in dying neurons after excitotoxic stimulation, and agents that reduce intracellular zinc concentration can provide neuroprotection [47]. However, the uptake of $Zn^{2+}$ did not account for the excitotoxicity-induced downregulation of the proteasome activity since the membrane-permeable $Zn^{2+}$ chelator N,N,N′,N′-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) was without effect (Supplementary Figure 2). This contrasts with the role of $Zn^{2+}$ in the ischemia-induced impairment of the UPS in the CA1 region of the hippocampus [48]. This discrepancy may be due to the differences between the two experimental models used. Overactivation of the NMDA receptors may lead, at least in part, to generation of excessive nitric oxide and reactive oxygen species, which in turn can mediate protein misfolding by S-nitrosylation or covalent reaction of NO with specific thiol groups [49]. Nonetheless, these mechanisms are not likely to be involved in the downregulation of the proteasome activity under excitotoxic conditions since the NOS general inhibitor L-NMMA was without effect. Furthermore, downregulation of the proteasome activity was observed in the presence of the antioxidants Vitamin E and GSH, suggesting that nitrosative and oxidative stress do not contribute to the impairment of proteasome activity (Supplementary Figures 3 and 4).

Ubiquitination is a reversible reaction, and before polyubiquitinated proteins are degraded in the proteasome the ubiquitin molecules are removed by DUBs to be recycled [50]. Therefore, the percentage of ubiquitinated proteins present in the cell at each given time is not only determined by the activity of the proteasome, but also by changes in the activity of DUBs. We found that excitotoxic glutamate stimulation decreases DUBs activity as determined with a fluorogenic substrate. However, the activity of Uch-L1 was not altered under the same conditions, suggesting that the observed impairment of DUBs activity is not an overall effect, but involves specific DUBs such as those associated with the proteasome. The activity of Ubp6, for example, is enhanced by interaction with the proteasome [36]. The lack of effect of excitotoxic stimulation on Uch-L1 activity contrasts with the upregulation observed in response to a shorter (possibly non-toxic) stimulation with NMDA, which upregulated the activity of this DUB [51].

In conclusion, our results show that glutamate-induced excitotoxicity impairs proteasome and DUB activity in cultured hippocampal neurons through activation of non-synaptic NMDA receptors. Additional studies are required to identify the intermediates linking non-synaptic NMDA receptors activation and proteasome deregulation, which may be potential therapeutic targets in brain ischemia.

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